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# Antimicrobial Activity of Lactic Acid Bacteria against Toxigenic Fungi

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## Abstract

*Lactobacillus bulgaricus* $S_2$ was widely used in fermentation and preservation of food and it was assayed on their fungal inhibitory properties. The aim of this study was to isolate lactic acid bacteria from different habitats and screen these isolates against some mycotoxigenic fungi. The effect of lactic acid bacteria (LAB) on growth of a mycotoxin- producing toxigenic fungi was assayed. Assays were carried out by modified overlay method. *Lactobacillus bulgaricus* $S_2$ assays showed growth inhibition of the mycotoxin-producing *Aspergillus flavus. Lactobacillus bulgaricus* $S_2$ isolated from sheep milk and selected for its technological properties, showed highest fungal inhibition of the micro-organisms assays. The use of antifungal LAB with excellent technological properties rather than chemical preservatives would enable the food industry to produce organic food without addition of chemical substances.

## Article Info

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## Keywords

Antimicrobial activity,
Lactic acid bacteria,
*Lactobacillus bulgaricus*,
Toxigenic fungi.

## Introduction

Molds and yeasts cause major problems in food and feed as spoilage organisms. They are particularly important because they produce mycotoxins (Pitt and Hocking, 1997). Bio-preservation, the use of microorganisms to preserve food and feed, has been considered as an alternative to the use of chemical preservation in the expectation that they could be safer (Strom *et al.*, 2002).

Lactic acid bacteria (LAB) are a broad group of Gram positive, catalase-negative, non-spore forming rods and cocci, usually non-motile, that utilize carbohydrates or fermented and form lactic acid as the major end product (Aguirre and Collins, 1993). With occasional exception, they are aero-tolerant anaerobes. Lactic acid bacteria are widely distributed in several raw materials (milk, meat and flour), soil, silage and waste products (Holzapfel *et al.*, 2001)

Most representatives of LAB do not pose any health risk to man and are designated as "Generally Recognized as Safe" (GRAS). They have commonly been used as a starter culture and play an essential role in manufacturing

of a wide variety of fermented food such as curd, cheese, yoghurts, dry sausages, beers and sour doughs (Singh and Prakash, 2009).

Lactic acid bacteria (*Lactobacillus fermentum* and *Lactobacillus rhamnosus*) and *Saccharomyces cerevisiae* showed growth inhibition of the mycotoxin-producing *Aspergillus. L. rhamnosus* O236, isolated from sheep milk and selected for its technological properties, showed the highest fungal inhibition of the microorganisms assays (Muñoz *et al.*, 2010). The LAB isolated from five indigenously fermented cereal gruels inhibited at least one aflatoxin-producing fungal isolate to varying extents (Onilude *et al.*, 2005).

Magnusson *et al.*, reported that *Lactobacillus coryniformis* subsp. *Coryniformis* strain Si3 produced proteinaceous compounds with a broad spectrum inhibitory action against several molds such as *Aspergillus fumigatus, A. nidulans, Penicillium roqueforti, Mucorhiemalis, Talaromyces flavus, Fusarium poae, F. graminearum, F. culmorum* and *F. sporotrichoides* (Magnusson *et al.*, 2003).

Various mechanisms have been suggested to be responsible for the inhibitory effects of the bacteria on fungal growth, such as nutritional competition, secondary metabolites, pH or combinations of these mechanisms (El-Nezami *et al.*, 2002). The inhibitory activity towards molds could be considered a characteristic for the selection of LAB used as starter cultures in grain ensiling of animal food in order to prevent avian fungal infection (Muñoz *et al.*, 2010).

The aim of this study was to isolate lactic acid bacteria from different habitats and screen these isolates against some mycotoxigenic fungi. The effect of *L. bulgaricus* $S_2$ was assessed with excellent industrial and health properties on growth, cell morphology and aflatoxins production of *A. flavus*.

## Materials and Methods

Twenty-one bacteria were isolated from human milk, sheep milk, yoghurt, pasteurized milk and fermented food "pickles". All isolates of LAB were selected and purified on De Mann, Rogosa and Sharpe (MRS) medium after incubation for 48 hours at 30$^{o}$C and pH 6.

All isolates of LAB were screened for antimicrobial activity against *Aspergillus niger*, *Penicillium* sp., *A. flavus, Aspergillus ochraceus* and *Fusarium* sp. using modified overlay method that described by Magnusson and Schnurer (2000). The inhibitory effect was determined on plates using an overlay technique; the potentially inhibitory microorganisms were inoculated first on solid agar (using MRS media) and then test fungi was inoculated on top (with soft PDA media).

The maximum antifungal activity of the isolates in this study was selected and identified to genus level initially by morphological and physiological tests and according to Bergey's Manual of Systematic Bacteriology (Holt *et al.*, 1994; Sharpe *et al.*, 1979)

Effect of the active material on aflatoxins production of *A. flavus* was studied using thin-layer chromatography (TLC) and Chromato-Vue Cabinet (Model CC- 60, UVP Inc, an Gabried, California) under visible light or short wavelength UV light (254 nm) and long wavelength (366 nm).

## Results and Discussion

Only twenty-one bacterial isolates were obtained from the normal habitats of lactic acid bacteria. Seven bacterial isolates were obtained from human milk, whereas 3 isolates were found in sheep milk. Moreover, 4, 3 and 4 isolates were recovered from yoghurt, pasteurized milk and fermented pickles, respectively. Screening of all isolates of LAB for antimicrobial activity revealed that the highest antimicrobial activities were against *A. niger*, *Penicillium.* sp., *A. flavus* (Table 1; Figures 1 and 2), *A. ochraceus* and *Fusarium* sp. No activity was observed against *A. ochraceus* and *Fusarium porotrichoides* (Table 1).

The isolate $S_2$ was the most active isolate and it was characterized and identified through physiological and biochemical tests. The selected isolate *L. bulgaricus* $S_2$ was tested against different toxigenic fungi (*Alternaria* sp., *Trichosporon mycotoxinivorans* and *Cladosporium* sp.) using well diffusion method (Table 2) according to Harris *et al.*, (1989). The result in table (3) shows that the inhibitory agent of the selected isolate inhibited the fungal growth and spore formation of *A. flavus*. Scanning electron micrographs revealed structural changes in the morphology of *A. flavus* during any of the inhibitory experiments (Figures 3 and 4). The treated conidiophores had rough surface and was distorted and the spores appeared with normal shape in control *A. flavus* compared to treated cells which was elongated and appeared hollow and quantity of aflatoxins production was decreased (Figure 5).

Nearly 25% of the European diet and 60% of the diet in many developing countries consist of fermented foods (Stiles, 1996). In addition, poultry for human consumption is generally fed on cereals or their products (Pinho and Furlong, 2000). Arqués *et al.*, (2015) studied antimicrobial activity of lactic acid bacteria in dairy products and gut and their effect on pathogens.

There was a predominance of *Aspergillus* species during storage period of coffee beans (Arqués *et al.*, 2015). Therefore, growth of the fungus in cereal crops could affect humans not only after consumption of infected cereals, but also after chicken consumption. In addition, the presence of toxigenic molds represents a potential risk of mycotoxin contamination especially while considering the worldwide increased use of herbal products as alternative medicine (Bugno *et al.*, 2006).

Inhibition of mycotoxigenic fungi is necessary in order to avoid toxin formation in food and feed. According to our results, natural control of the microflora could be realized by beneficial microorganisms. The number of publications on antifungal LAB is still low (Schnürer and Magnusson, 2005). LAB produce a variety of antimicrobial compounds. Various mechanisms have been suggested to be responsible for the inhibitory effects of the bacteria on fungal growth, such as nutritional competition, secondary metabolites, pH or combinations of these mechanisms (El-Nezami *et al.*, 2002). *L. acidophilus* and *Bifidobacterium animalis* strains are able to detoxify the mycotoxins. Both species can be used for the production of probiotic fermented foods. Therefore, our findings may contribute to the development of strategies for the detoxification of contaminated plant derived products with these toxins by use of LAB (Fuchs, 2008).

Other authors have suggested that aflatoxin biosynthesis was inhibited by LAB but that the bacteria were not efficient enough to remove aflatoxin from the medium (Thyagaraja and Hosono, 1994). Our studies confirm previous studies demonstrating the inhibitory activity by LAB against a mycotoxin-producing fungus. *L. bulgaricus* $S_2$ isolated from sheep milk and selected for its technological properties showed highest fungal inhibition of the microorganisms assayed.

**Table.1** Screening of the recovered bacterial isolates for antimicrobial activity against some tested fungi using modified overlay method

| Isolate code | Mean diameter of inhibition zone (mm) ± SD | | | | |
|---|---|---|---|---|---|
| | ***A. niger*** | ***A. ochraceus*** | ***A. flavus*** | ***Penicillium* sp.** | ***Fusarium sporotrichoides*** |
| **$H_1$** | 10.0±2.0 | 0.0±0.0 | 18.0±2.6 | 7.6±1.5 | 0.0±0.0 |
| **$H_2$** | 16.3±2.3 | 0.0±0.0 | 9.0±1.0 | 19.3±1.1 | 0.0±0.0 |
| **$H_3$** | 24.6±6.4 | 0.0±0.0 | 23.3±3.5 | 18.3±2.0 | 0.0±0.0 |
| **$H_4$** | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 |
| **$H_5$** | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 |
| **$H_6$** | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 |
| **$H_7$** | 15.6±4.0 | 0.0±0.0 | 19.3±5.1 | 8.0±2.6 | 0.0±0.0 |
| **$S_1$** | 8.0±1.0 | 0.0±0.0 | 2.6±1.1 | 2.3±0.5 | 0.0±0.0 |
| **$S_2$** | 34.0±4.5 | 0.0±0.0 | 24.6±0.5 | 29.0±1.0 | 0.0±0.0 |
| **$S_3$** | 9.3±0.5 | 0.0±0.0 | 12.6±2.0 | 3.0±1.7 | 0.0±0.0 |
| **$Y_1$** | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 |
| **$Y_2$** | 3.6±1.5 | 0.0±0.0 | 2.6±0.5 | 1.6±0.5 | 0.0±0.0 |
| **$Y_3$** | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 | 3.3±0.5 | 0.0±0.0 |
| **$Y_4$** | 3.6±0.5 | 0.0±0.0 | 3.0±1.7 | 0.0±0.0 | 0.0±0.0 |
| **$L_1$** | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 | 1.3±0.5 | 0.0±0.0 |
| **$L_2$** | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 |
| **$L_3$** | 0.0±0.0 | 0.0±0.0 | 2.3±0.5 | 0.0±0.0 | 0.0±0.0 |
| **$F_1$** | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 |
| **$F_2$** | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 |
| **$F_3$** | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 |
| **$F_4$** | 2.6±1.1 | 0.0±0.0 | 5.3±0.5 | 0.0±0.0 | 0.0±0.0 |

$H_1$ to $H_7$: Human Milk; $S_1$ to $S_3$: Sheep Milk; $Y_1$ to $Y_4$: Yoghurt; $L_1$ to $L_3$: Pasteurized Milk; $F_1$ to $F_4$: Fermented Pickle.

**Table.2** Screening of the selected bacterial isolate $S_2$ for antimicrobial activity against some toxigenic fungi using well-diffusion method

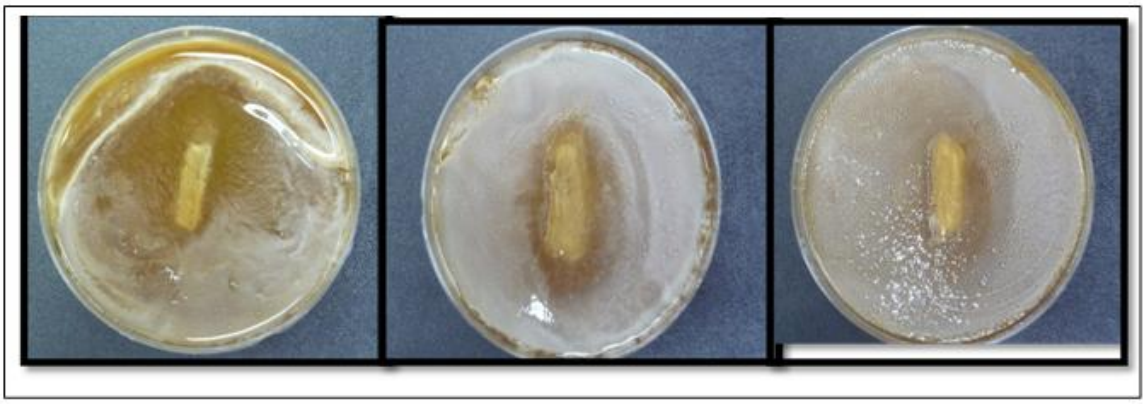
| The tested toxigenic fungi | Mean diameter of inhibition zone (mm) ± SD |
|---|---|
| *Aspergillus niger* | 13.6 ±1.5 |
| *Aspergillus flavus* | 19.0 ±1.0 |
| *Penicillium* sp. | 15.3 ±4.5 |
| *Alternaria* sp. | 28.6 ±1.1 |
| *Trichosporon mycotoxinivorans* | 22.3 ±2.0 |
| *Cladosporium* sp. | 17.3 ±1.1 |

**Table.3** Effect of the active material on number of colony of *Aspergillus flavus*

| Effect of the active material on number of spore/ml | |
|---|---|
| Normal | Treated |
| $2 \times 10^4$ ±3.6 | $2.2 \times 10^2$ ±1.0* |

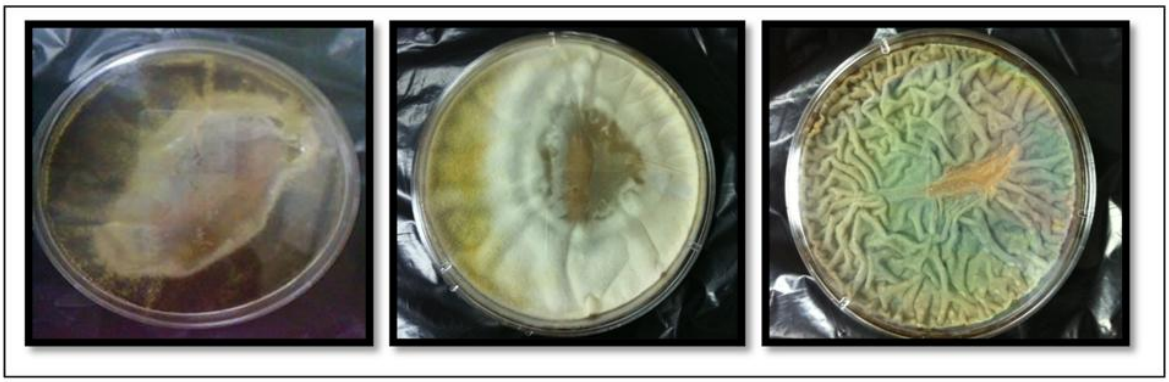
*: Significant at *p*-value ≤ 0.05

**Fig.1** Antimicrobial activity of the isolated bacterium S2 against toxigenic fungi using modified overlay method



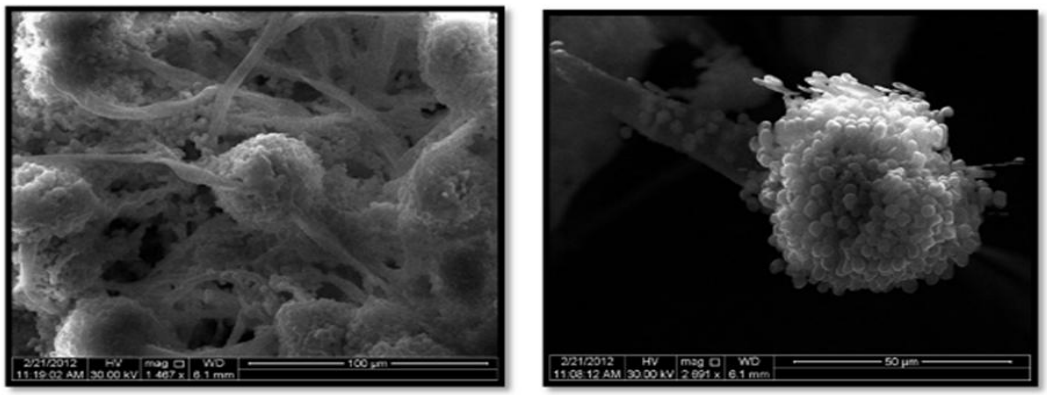
*Aspergillus niger* *Penicillium* sp. *Aspergillusflavus*

**Fig.2** Antimicrobial activity of isolated bacterium H3 against toxigenic fungi using modified overlay method



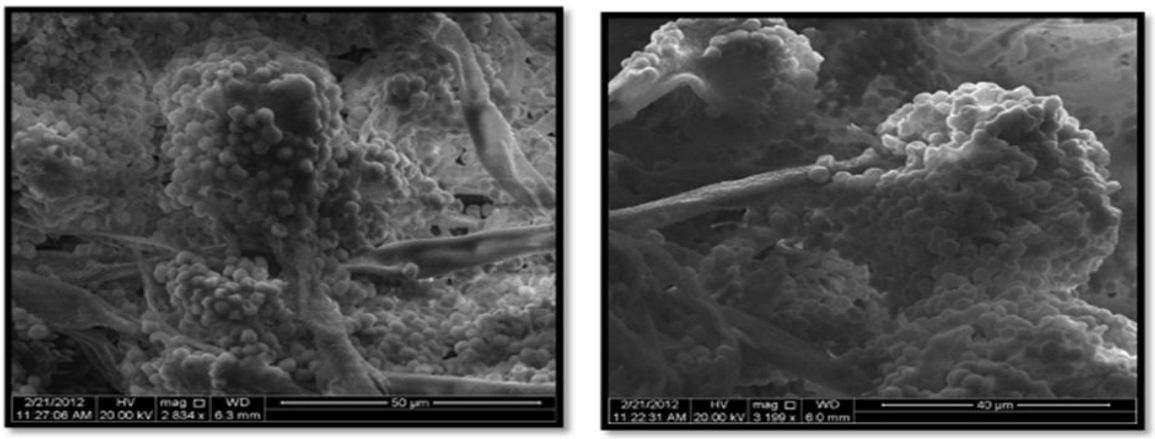
*Aspergillus niger* *Aspergillus flavus* *Penicillium* sp.

**Fig.3** *Aspergillus flavus* (conidiophores and spores) under scanning electron microscope (A and B) with different magnification after 48 hours at 28°C (untreated)



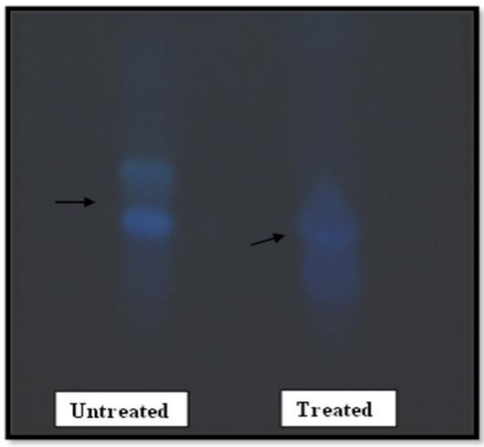
A B

**Fig.4** Effect of antimicrobial agent on morphology of *Aspergillus flavus* (conidiophores and spores) under scanning electron microscope (A and B) after 48 hours at 28°C



A B

**Fig.5** Aflatoxin detection at UV254 in untreated and treated *Aspergillus* extract using UV light



Probiotics are usually isolated from the gastrointestinal tract of humans and animals. The search of probiotics in human milk was a recent field of research, as the existence of the human milk microbiome was discovered only about a decade ago; Quilodrán-Vega *et al.,* (2016) isolated several lactic acid bacteria from swine milk and evaluated them for their potential as probiotics, they showed that *L. curvatus* TUCO-5E is a good candidate to study *in vivo* the protective effect of probiotics against intestinal infection and damage induced by *Salmonella* infection in the porcine host (Quilodrán-Vega *et al.,* 2016).

The inhibitory activity of lactobacilli against molds could be due to different factors. It is worthy to mention that the obtained 21 bacterial isolates were screened for their antimicrobial activity against some mycotoxigenic fungi using modified overlay method described by Magnusson and Schnurer (2000). Onilude *et al.,* (2005) and Munoz *et al.,* (2010) used the modified overlay method to detect the antimicrobial activity of lactic acid against aflatoxin-producing fungi.

Temperature and incubation period were essential factors that modulate LAB growth and significantly affect the amounts of antifungal metabolites produced by LAB (Batish *et al.,*1997) The studies carried out by Sathe *et al.,*(2007) demonstrated that antifungal activity of *Lb. plantarum* CUK501 was maximal at 30°C, when the culture was at the end of its logarithmic phase. Batish *et al.,* (1990) observed that the antifungal activity of a *L. acidophilus* strain was maximal at 30°C after 48 h incubation, whereas increasing the incubation period resulted in a lower antifungal activity. These "antimycotoxinogenic" metabolites could also be produced during LAB growth (Dalié, *et al.,* 2010). Growth, cell numbers, morphological characters and toxin production of *A. flavus*, treated with $S_2$ filtrate were determined and compared to that obtained for untreated *Aspergillus* (control). The growth and the number of the cells were decreased by the antimicrobial agent $S_2$. The fungal morphology was changed and the quantity of aflatoxin production was decreased. Our results are in agreement with those previously observed by Onilude *et al.,* (2005) that show the lactic acid bacteria isolates to have effect on the different *Aspergillus* species prior to the sporulation of the latter. *Lactobacillus fermentum* RS2 was observed to exhibit maximum inhibition on mycelial development for most of the *Aspergillus* species while *Lactobacillus* spp. had the lowest. Thyagaraja and Hosono (1994) suggested that aflatoxin biosynthesis was inhibited by LAB but the bacteria were not efficient enough to remove aflatoxin from the medium. The interaction between mycotoxin producing fungi and other microorganisms is a common phenomenon in nature that can affect fungal growth and/or production of mycotoxins (Thyagaraja and Hosono, 1994).

The use of antifungal LAB instead of chemical preservatives would enable the food industry to produce organic food without addition of chemical substances. In addition to the already known excellent properties of LAB they could enhance the nutritional value and prolong conservation of food (Hassan and Bullerman, 1997).

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